Pl. Syst. Evol. 255: 193–214 (2005)
DOI 10.1007/s00606-005-0314-8

Plant Systematics and Evolution
Printed in Austria

How selfing and intra- and interspecific crossing influence seed set, morphology and ploidy level in *Euphrasia*: An experimental study of species occurring in the Alps of Switzerland

B. Liebst and **J. Schneller**

Institut für Systematische Botanik, Universität Zürich, Zürich, Switzerland

Received November 8, 2004; accepted February 19, 2005
Published online: August 11, 2005

Abstract. Annual alpine species rely on selfing rather than on cross-pollination for successful reproduction. However, insect visits may occasionally cause cross-pollination not only within but also between closely related species. The aim of this study was to investigate four species of *Euphrasia* for their efficiency in spontaneous selfing and their success in intra- and interspecific crossing. We used the seed sets that followed spontaneous selfing and artificial cross- and selfpollination to measure the breeding success. We compared the morphological characters of species and hybrids and determined their ploidy level using flow cytometry. We verified the hybridogenous origin of plants resulting from interspecific crosses using RAPD banding patterns. While spontaneous seed set was high in the two small-flowered species, seed set in the large-flowered species was small and affected by external circumstances. We obtained F1 and F2 hybrids from interspecific crosses of two diploid species and detected polyploid individuals in both generations.

Key words: *Euphrasia christii*, *E. hirtella*, *E. minima*, *E. rostkoviana* (Orobanchaceae), Germination, hybridization, intra- and interspecific cross-pollination, morphology, polyploidization, selfing.

Introduction

An essential step in the sexual reproduction of a plant is the successful pollination of its flowers either by out-crossing or by selfing. While perennial plants may persist without yearly reproduction or may rely on vegetative propagation, annual species must reproduce at a site every year to persist (Reynolds 1984) or must develop a seed bank. In an alpine ecosystem, low temperatures and a short, unpredictable growing season are limiting factors for plant growth and reproduction (Bell and Bliss 1980). Rapid pollination shortens the time until the seeds mature and therefore minimises the risk of seed loss due to the early onset of winter. Although it has been shown that low levels of insect diversity and abundance in alpine ecosystems do not reduce cross-pollination in every case (Bingham 1998), it may be expected that annuals reproduce more successfully if they are independent of pollinators and may self-pollinate, at least to some degree, immediately after or even before the opening of their flowers.

Annual species commonly do not represent more than 2% of the total alpine flora and

become increasingly rare with increasing altitude (Körner 1999). When we checked European and Swiss floras for annual alpine plants (Hartl 1974, Hegi et al. 1977, Yeo 1978, Lauber and Wagner 1996), we found at least four species of the genus *Euphrasia* reaching altitudes of between 2500 and 3200m and a further six species in lower alpine regions, including the treeline-ecotone (definition according to Körner (1999)). It is believed that all species of the genus are self-fertile but that they differ in their ability to self. Richards (1996) states that the amount of selfing from within-flower pollination depends on the degree of separation that occurs between pollen donation from anthers and pollen reception by stigmas in time and space. Because the large-flowered species of *Euphrasia* are proterogynous and the positions of their stigmas and anthers do not support selfing, it is assumed that they are primarily adapted to pollination by insects. However, while some authors state that members of this group of *Euphrasia* cannot self, others suggest that they are self-pollinated to some extent (Yeo 1966). The small-flowered *Euphrasia* species are initially hermaphroditic, and self-pollination appears to occur regularly at the beginning of anthesis (Müller 1881, von Wettstein 1896). Experimental work conducted recently supports the assumption that small-flowered *Euphrasia* species are predominantly selfing (Molau 1993, Gomez 2002).

Although studies concerning selfing in alpine *Euphrasia* species have been done, studies concerning cross-pollination are lacking. Cross-pollination occurs not only within, but also between *Euphrasia* species. Between-species pollination in *Euphrasia* is of particular interest, because it is assumed to be an important factor in the variation of the genus and because the evolution of new species may be a result of hybridization (Yeo 1978, Vitek 1986). Putative hybrids found in natural populations (von Wettstein 1896; Hartl 1974; Vitek 1985, 1986) and artificial interspecific crossings, especially of British *Euphrasia* species (Yeo 1976), have revealed high interfertility between diploid species. Diploid-tetraploid hybridization as a source of new *Euphrasia* species has been discussed by Yeo (1956).

In the Alps, both the distribution areas and the flowering seasons of many *Euphrasia* species overlap, and a number of hybrids that probably originated from homoploid species and from parents with different ploidy levels have been described (Vitek 1985, 1986). However, whether an individual plant has a hybridogenous origin or what the parental species of a hybrid are, often cannot be determined. As far as we know, hybridization experiments including alpine *Euphrasia* species have not been done, nor has the ploidy level of putative hybrids found in the Alps been determined. An example of an alpine *Euphrasia* species probably of hybridogenous origin may be the most common *Euphrasia* species in European high mountains, the tetraploid *E. minima*. Based on morphological and ecological characteristics of the two diploid alpine species *E. christii* and *E. hirtella* and – except for the flower size – intermediate characteristics of *E. minima*, it seems probable that *E. minima* descended from forms of *E. christii*, tending to alpine dwarf forms, and from *E. hirtella* (Vitek 1986).

Our study focused on the pollination system and the seed set of four *Euphrasia* species occurring in the Alps of Switzerland: *E. christii*, *E. hirtella*, *E. minima* and *E. rostkoviana*. We used the seed set resulting from selfing and artificial self- and cross-pollination to answer the following questions: (1) Does the seed set resulting from selfing differ from the seed set resulting from artificial self- or cross-pollination within the species? (2) Does the seed set following selfing differ between small- and large-flowered species?

We investigated the interfertility between two diploid species (*E. christii* and *E. hirtella*) and between a tetraploid and a diploid species (*E. minima* and *E. rostkoviana*), the ploidy level of the hybrids and the morphological differences between the hybrids and their parental species, to answer the following questions: (3) Does interspecific crossing result in seed set, and is there a difference in seed set between the

diploid species pair and the species pair with different ploidy levels? (4) How do hybrids differ morphologically from the parental species? (5) Are the F1 and the F2 hybrids fertile? (6) Does the ploidy level of the hybrids differ from that of the parental plants? (7) Can the hybrids be distinguished from the parental species by molecular markers?

Material and methods

Species. Four species of section *Euphrasia*, subsection *Ciliatae* Joerg., were included in this study: *Euphrasia hirtella* and *E. rostkoviana* (series *Grandiflorae* Wettst.), *E. minima* (series *Parviflorae* Wettst.) and *E. christii* (series *Alpinae* Rothmaler) (von Wettstein 1896, Yeo 1978). While *E. rostkoviana*, *E. minima* and *E. hirtella* are widespread in the Alps, *E. christii* is restricted to alpine regions in southern Switzerland and northern Italy. The species' altitudinal distributions in the Alps range from intermediate to high regions for *E. rostkoviana* (highest record 2600 m), from 1500 to 2500 m for *E. minima* (highest record in Switzerland 3250 m), from subalpine to alpine regions for *E. hirtella* (highest record in the Alps 2400 m (E. Vitek, Wien, personal communication)) and from 1280 to 2500 m for *E. christii* (von Wettstein 1896, Braun-Blanquet and Rübel 1928, Hess et al. 1972, Hartl 1974, Yeo 1978, Käsermann and Landergott 1999). Where distribution areas and flowering time of *Euphrasia* overlap, interspecific hybrids are to be expected. With regard to the species included in this study, hybrids *E. minima* × *E. hirtella*, *E. rostkoviana* × *E. minima*, *E. hirtella* × *E. rostkoviana* and *E. minima* × *E. christii* have already been described (von Wettstein 1896, Hartl 1974, Vitek 1986).

Chromosome counts had been done for all four species of interest ($2n = 22$ for *E. christii*, *E. hirtella* and *E. rostkoviana*, $2n = 44$ for *E. minima*) (Greilhuber et al. 1984; Vitek 1985, 1986; Vitek and Kiehn 1990).

All the *Euphrasia* plants used in this study were grown from seeds in the Botanical Garden of the University of Zurich. For the P generation, we collected seeds from 20 plants of *E. christii* and from twelve plants of *E. hirtella* at the same location in the Swiss Alps (Chastelberg above Simplon Dorf, Canton Valais, 2197 m, 648 760/117 540). Seeds from 16 plants of *E. minima* and 20 plants of *E. rostkoviana* were collected at the Oberalppass (Canton Uri, 2044 m, 693900/168650) at the same time (September 2000). Seeds for the F1 and the F2 generations were collected after successful pollination and fruit set. The seeds of each plant were stored separately in a refrigerator at about 5° C.

Most recent classification systems propose *E. rostkoviana* as a subspecies of *E. officinalis* (Silverside 1991) and *E. christii* as a subspecies of *E. alpina* (Vitek 1986). In this paper, we followed the nomenclature of "Flora der Schweiz" (Hess et al. 1972).

Sowing and cultivation techniques. Small pots (fertil pot, Fertil S.A., 88120 Le syndicat, France), reduced to a cylinder with a diameter of 4 cm, were filled with a mixture of topsoil, peat and quartz sand (3:4:1). The sowings for all generations were each done in November and December. In each pot, three seeds were sown in such a way that they formed the corners of an isosceles triangle with a side length of about 2.5 cm. For the P generation, at least nine seeds from one plant were sown. For later generations, we sowed all the seeds of a fruit, but never more than three in a pot. The sowing pots for the P generation (2000) were kept damp at 5° C in a refrigerator with a glass-door and were rearranged every eight days until germination occurred in March. The pots for the F1 generation (2001) were kept in a vernalisation chamber (4° C, 16 hours artificial illumination). Within three weeks, most *E. rostkoviana* and *E. minima* seeds and a large number of *E. christii* and *E. hirtella* seeds had rotted. This rotting was stopped by using a fungicide (Chinosol, AlliedSignal Riedel-de Haën AG) and the remaining seedlings were used for the experiment. The sowing pots for the F2 generation (2002) were stored outside during winter. No seeds rotted and germination took place in March 2003. After germination, the treatment was the same for all generations. The pots were placed in an unheated greenhouse, and three seeds of *Lolium perenne* were sown as hosts at a distance of 1 cm from each of the *Euphrasia* seedlings. When the pot walls were penetrated by the roots, they were planted in larger plastic pots (diameter 8 cm). The use of this method made it possible to avoid seedling death caused by nettling. Nevertheless, a large number of plants died before flowering or later on, probably as a result of insect attacks (aphids, sciarid larvae) or because of high temperatures. The temperature in the greenhouse

depended greatly on the outside temperature and ranged from 5° C in late winter to 35° C in summer.

In addition to daylight, artificial illumination was provided for 16 hours a day by fluorescent lamps (Osram L36, W 72-965, Biolux fluorescent lamps at about 40 cm distance from the largest plants).

Pollination technique. At least one plant in each pot was used in the pollination experiment, in order to have a sufficient number of plants with the treatments shown in the pollination scheme (Fig. 1). Artificial pollination was started when five or more plants of a species began to flower. Prior to pollination, the flowers had to be emasculated, to avoid selfing.

All treatments had to be done under a stereomicroscope (Wild M3C, Heerbruck, Switzerland) because of the small size of the flowers (*E. minima* 4.4–5 mm, *E. christii* 7–11 mm, *E. hirtella* 5.5–7 mm, *E. rostkoviana* 8–12 mm). The pots containing the plants were laid on a small sandbag so that the flowers were in a suitable position for emasculation and pollination. *Euphrasia hirtella* and *E. minima* were emasculated before the buds opened or when the flowers just had opened, because pollination can take place in a very early stage of the anthesis, even within the buds. The upper lip was split lengthways using a fine needle, without damaging the style. With two forcepses, parts of the upper lip were removed until the filaments and the stigma were visible. The four filaments were then removed using small scissors (Fine Science Tools, No. 15001–08) and the stamens on the left and right sides of the stigma were carefully pulled apart, to detach the hairs connecting the pollen sacs. The flowers of *E. christii* and *E. rostkoviana* were emasculated in their female phase, after the buds had opened. Before the emasculated flowers were pollinated, the stigmas were checked carefully on pollen grains.

For intraspecific and interspecific cross-pollination, dehiscing anthers from 3–5 plants were cut off, and the pollen grains were shaken onto a slide. A small piece of nylon thread was used to pollinate the flowers. For artificial self-pollination, dehisced anthers either of the same flower or of a different flower from the same plant were cut off, and the pollengrains were transferred directly to the stigma of the flower to be pollinated. In all cases, the entire stigmatic surface was covered with pollen. If possible, consecutive flowers on the main stem were chosen for pollination. The order of the different pollinations of a plant was chosen at random.

The efficiency of self-pollination was tested at the same plants which were used for inter- and intraspecific crossing (Fig. 1) and additionally at plants which were used for spontaneous self-pollination only. In both cases, the buds were marked at their bracts without further treatment. About ten plants of each species were used exclusively for selfing. After marking one to two buds of each plant, the plants were enclosed and not touched during anthesis. In the flowers of these plants, pollination only could occur when in the hermaphroditic phase the stigma was in a position which allowed for catching pollen from the anthers. This kind of pollination is referred to as "spontaneous selfing". In the plants which were used for selfing and intra- and interspecific crossing, the position of the plants and consequently of the flowers used for selfing has changed during the artificial pollinations, thus possibly supporting self pollination. Therefore this kind of pollination is referred to as "supported selfing".

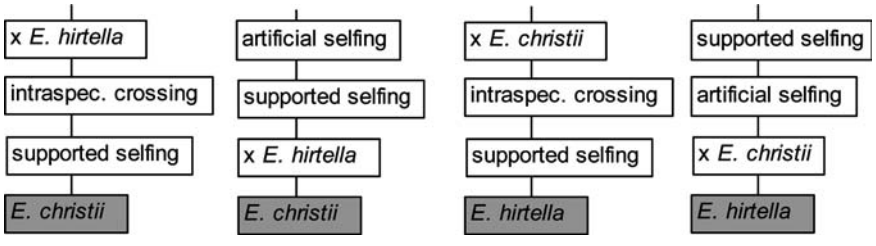

Fig. 1. Pollination scheme for the P generation of *E. christii* and *E. hirtella*. At each plant (given in grey), three flowers have been pollinated according to the scheme. Pollinations of *E. minima* and *E. rostkoviana* have been done in the same way. Pollinations of the F1 and F2 generation of *E. christii* and *E. hirtella* are described in the text

Table 1. Characters and indices for morphological studies

Definition	Codification	Abbreviation
1. Qualitative characters		
Colour of corolla	1 white	white
	2 yellow	yellow
	3 changing colour from yellow to white	yellow to white
Stem hairs	1 predominantly multicellular glandular hairs	pred glandular
	2 eglandular and glandular hairs (glandular hairs ≤ eglandular hairs)	glandular/ eglandular
	3 only eglandular hairs	only eglandular
Leaf hairs	1 predominantly multicellular glandular hairs	pred glandular
	2 eglandular and glandular hairs (glandular hairs ≤ eglandular hairs)	glandular/ eglandular
	3 predominantly eglandular hairs (glandular hairs ≤ eglandular hairs)	pred eglandular
	4 only eglandular hairs	only eglandular
	5 leaves subglabrous to glabrous	glabrous
For the discriminant analysis, nominal variables were transformed to dummy-variables.		
2. Quantitative characters		
Flowers		
• Length upperlip	mm	Upperlip
• Length corolla-tube	mm	Tube
• Length teeth calyx	mm	Calyx
Leaves		
(measurements refer to the largest cauline and the largest floral leaf of a plant)		
• Angle of leaf base (cauline)		Ang_l_c
• Angle of apex tooth (cauline)		Ang_t_c
• Number of leaf tooth pairs (cauline)		Teeth_c
• Angle of leaf base (floral)		Ang_l_f
• Angle of top tooth (floral)		Ang_t_f
• Number of leaf tooth pairs (floral)		Teeth_f
3. Indices		
Ratio of calyx length to calyx width		In_calyx
Ratio of leaf length to leaf width (cauline)		In_leaf_c
Ratio of leaf length to leaf width (floral)		In_leaf_fl
4. Hybrids		
The first species in the hybrid name is the maternal species		

A colour code was used to distinguish the different treatments. The bracts of the pollinated flowers were tagged with a very small amount of waterproof paint (Waco acrylic dim paint, Hch. Wagner AG, 8105 Regensdorf (Switzerland)).

After the pollinations, the pots were enclosed in nylon mesh bags until the colour of the stigma of the pollinated flower had changed from white to brownish. Because bagging may change microclimatic conditions (Kearns and Inouye 1993), it was kept as short as possible (2–4 days).

Seed harvesting. To avoid seed loss, the ripening fruits were enveloped in small bags made of glassine paper, about two weeks after pollination. Because this procedure is very time consuming, not all of the fruits were enveloped. About three weeks after pollination, for each species and each treatment, the number of non-developed fruits was determined. All fruits resulting from the artificial pollination and all fruits marked as resulting from spontaneous or supported pollination were collected when they started to open. Seeds and ovules were counted under the stereomicroscope. All normally developed seeds and all seeds with abnormal looking testa or small contents were stored dry at room temperature for about two weeks and then in a refrigerator at about 5° C.

Statistical analyses of the pollination experiment. For all species, the dependence of fruit set on pollination treatments was tested by Pearson's chi-square test. Due to "expected values" being smaller than five, the Yates' correction was also applied. Both Pearson's tests, with and without Yates' correction, led to the same results. With regard to the interspecific crossings, the chi-square test was applied to test the dependence of fruit set on the maternal plant.

For each fruit, the relative seed set (rss, seed:ovule) was calculated. Because data relating to relative seed set did not fit normal distribution even after transformations, and/or because assumption of homogeneity of variance was not fulfilled, nonparametric tests were used for comparisons between pollination treatments.

To compare the relative seed set resulting from artificial cross-pollination with the relative seed set resulting from artificial self-pollination, the Wilcoxon signed-rank test was applied. The same test was used to compare the relative seed set resulting from artificial self-pollination with the relative seed set resulting from supported self-pollination. No test was applied on seed set after interspecific crossing of the diploid *E. rostkoviana* and the tetraploid *E. minima*, because pollinations resulted in just one and four fruits respectively.

For each species, the mean value of rss was calculated for the plants which were used for spontaneous selfing only.

Morphological investigations. All plants were pressed and dried for morphological and RAPD analyses. Because none of the seeds resulting from the interspecific crossing of *E. rostkoviana* and *E. minima* germinated, only the species *E. christii* and *E. hirtella* could be used to study the morphological characters of parental plants and hybrids. Flower colours were determined at living plants. Quantitative flower characters were measured in one fully developed flower per plant after boiling up. Measurements were taken under a stereomicroscope at magnifications of 6.4x and 10x. Prior to pressing

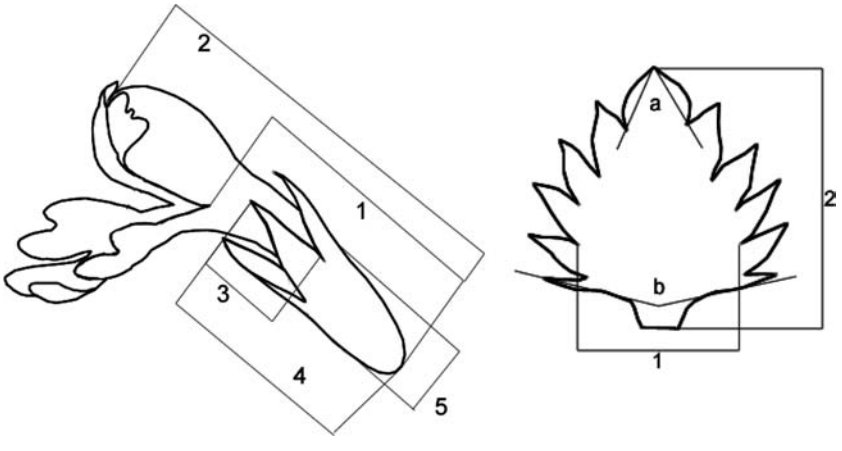

Fig. 2. Flower: (1) Length corolla tube, (2) length upper lip, (3) length teeth calyx, (4) length calyx, (5) width calyx. Leaf: (1) Width leaf, (2) length leaf, (a) angle of top tooth, (b) angle of leaf base

and drying, the largest cauline leaf and the largest bract of each plant were mounted on a sheet of paper using transparent adhesive tape. The sheet was then photographed with a digital camera. Leaf length and width and the angles of the leaf base and the apex tooth were determined by the program Adobe Photoshop 7.0, using a ruler as reference. The length of the glandular hairs on the stem and leaves was determined by comparison with eglandular hairs on the same plant. Nine metric variables, three indices and three nominal variables were used for morphological analyses of a total of 48 plants (Table 1, Fig. 2).

For statistical analyses, individuals of generations P, F1 and F2 were pooled within each of the groups *E. christii* and *E. hirtella*. Hybrids from reciprocal crosses were regarded as separate groups, but within each group plants of all generations inclusive plants resulting from back-crossing were pooled because of the small sample sizes. The group *E. christii* × *E. hirtella* consisted of three F1 hybrids, eleven plants resulting from selfing of the F1 hybrids, one individual resulting from back-crossing of a F1 hybrid with *E. christii* and one individual resulting from back-crossing of *E. christii* with a F1 hybrid. The group *E. hirtella* × *E. christii* consisted of five individuals resulting from selfing of two F1 hybrids.

Statistical analysis of biometry. Means and coefficients of variation were calculated for quantitative characters and indices with respect to the four groups *E. christii*, *E. hirtella*, *E. christii* × *E. hirtella* and *E. hirtella* × *E. christii*. Discriminant function analysis was used to test whether these groups could be separated by morphological characters. Assumptions for discriminant function analysis are normal distribution within groups and equal population covariance matrices. Normal distribution within groups was tested using the Shapiro–Wilk's test.

Vouchers are deposited in the herbarium of "Z". Data matrices are available from the authors on request.

RAPD analyses. Eleven *E. christii* and 18 *E. hirtella* plants were used to identify RAPD fragments specific for either of the species. None of the F1 hybrids could be used for the RAPD analysis because they were wanted for the pollination experiment. Instead, ten F2 plants of hybridogenous origin were analysed.

Primer screening was performed using six individuals belonging to each species and 38 of altogether 80 primers (Operon Technologies, kits A-D). Twenty-six primers yielded in amplification products, but only five of them met the conditions of sufficient reproducibility and distinctly different fragments for both of the species. The reproducibility of the patterns was tested by repeated amplifications and by variation of the reaction mixtures (modification of $MgCl_2$ and/or DNA concentration).

DNA was isolated from dried leaf material using the Qiagen RNeasy MiniKit and the manufacturer's protocol, but the incubation time was extended to 20 min. Because *Euphrasia* leaves are very small, in many cases all the leaves of a plant were used to get enough dried material (about 20 mg, but in some cases less than 10 mg). The isolated DNA was stored at –20° C until amplification. The PCR reactions were performed by using a 12.5 µl reaction mixture containing 2 mM $MgCl_2$, 0.1 mM dNTP's, 0.2 µM oligonucleotide RAPD primers (Operon Technologies), 0.5 U Taq polymerase and 1.5 mM supplied polymerase buffer (Amersham Pharmacia Biotech) in sterilised aqua bidest. Thermal cycling was performed in a Genius thermocycler (Techne) using the following cycle profile: 3 min initial denaturation at 94° C followed by 43 cycles consisting of 30 sec at 94° C, 1 min at 39.5° C, 1.5 min at 72° C and 5 min final elongation at 72° C. The amplification products were separated in 1.5% (w/v) agarose gels in 1x TAE buffer at 65 V for four hours and subsequently stained in ethidium bromide (10 min) and washed in tap water (10 min). Gel documentation was done with the MultiGenius System (Syngene).

Ploidy analyses. The ploidy level of a total of 15 plants of the F1 generation and 31 plants of the F2 generation was determined by estimating the relative DNA content using flow cytometry.

Table 2. Number of fruits resulting from spontaneous self-pollination and artificial selfing and crossing, expressed as a percentage

	Supported selfing	Artificial selfing	Artificial crossing
E. christii	40.9	100.0	100.0
E. hirtella	100.0	100.0	100.0
E. minima	92.0	100.0	100.0
E. rostkoviana	90.5	100.0	92.9

For analysis, 2–3 young leaves per plant were removed, placed in a plastic bag and put in a refrigerator until analysis could be done either on the same day or the following day. Approximately 1 cm^2 of young leaf material was chilled on ice and chopped with a razor blade to release nuclei in a petri-dish containing 0.5 ml of nuclei isolation buffer (commercial Partec CyStain UV precise P, solution A). The homogenate was filtered through a 50 µm nylon mesh and incubated for 3 min on ice. Nuclei were stained with fluorescent dye DAPI (4'-6-diamidino-2-phenylindole, Partec CyStain UV precise P, solution B, about 2 ml) and were analysed after 30 s using a Partec Ploidy Analyser-II (UV excitation with a mercury arc lamp). Leaf material of *Pisum sativum* cv. Minerva Maple was run initially on each day of analysis as external standard. To account for variation in machine parameters between one day and another, the fluorescence of the samples was expressed as the proportion of the value for the standard run on the same day (relative fluorescence) (Husband and Schemske 1998).

Repeated analyses of different leaves of the same *Euphrasia* plant on the same day showed different peak positions in some cases. Furthermore, the younger leaves often showed higher fluorescence values than older leaves, but sometimes revealed a rapid decrease of fluorescence depending on the incubation time. Peak shifting occurred only slightly in the external standard *P. sativum*. To improve measurements, different isolation buffers were tested: a buffer containing $MgCl_2$, NaCl, Tris and Tritonx100 (Tim Sharpel, Jena, personal communication), CyStain UV Precise T isolation buffer and modification of the Partec Precise P isolation buffer either with mercapto ethanol, ascorbic acid or PVP. None of these buffers significantly improved peak shifting. The best results were obtained by using Partec CyStain UV precise P and by keeping short the incubation time. Because fluorescence may vary between the different leaves of a plant, the interpretation of the results has to be viewed with caution.

To calculate the mean relative fluorescence for the species, data from the generations F1 and F2 were pooled.

Results

Germination of the seeds. The sowings for the P generation resulted in germination rates of about 46% in *E. christii*, 15% in *E. hirtella*, 27% in *E. minima* and 21% in *E. rostkoviana*.

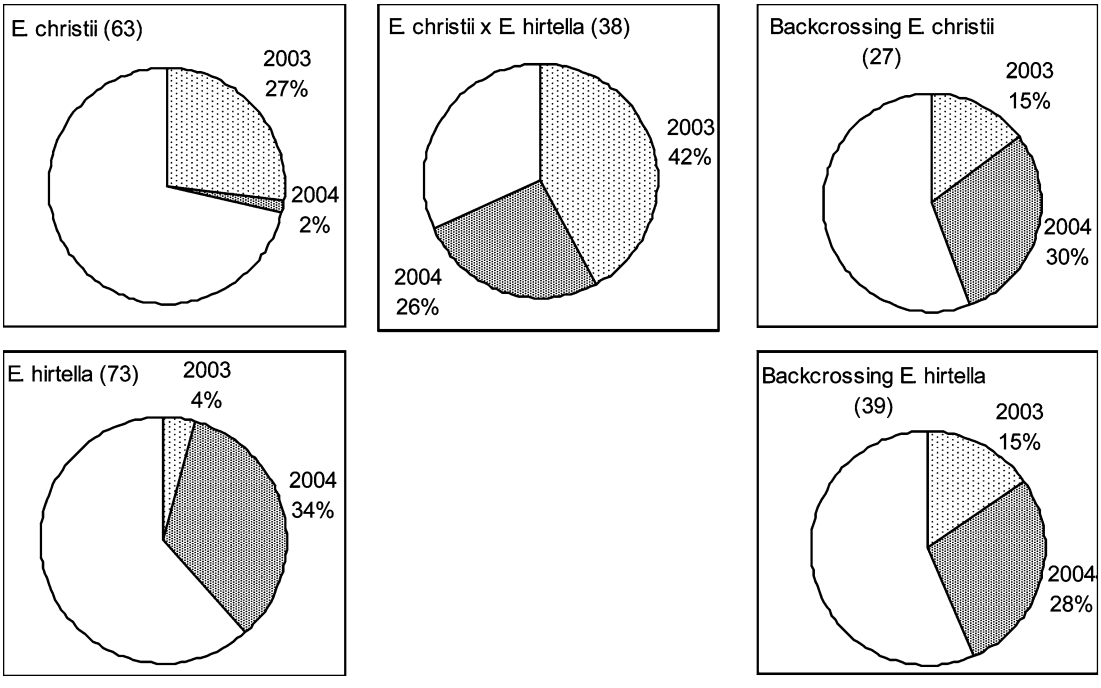

Fig. 3. Number of seeds (F2) which germinated in the spring after sowing (2003) and in the following year. Total number of seeds in brackets

Different germination rates and plant dying in an early development stage led to an unequal number of plants per species which could be used for the pollination experiment.

Most seeds resulting from pollinations of *E. minima* and *E. rostkoviana* (P generation) and a large number of seeds resulting from pollinations of *E. christii* and *E. hirtella* (P generation) rotted in the vernalisation chamber. Consequently, no germination rate of species and hybrids (F1) was determined.

Euphrasia christii, *E. hirtella* and their hybrids (F2) showed different germination rates within two years of observation (Fig. 3). A number of seeds which failed to germinate in March or April following sowing germinated in the ensuing spring. These seedlings were not included in the study.

None of the seeds resulting from interspecific crossing of *E. rostkoviana* and *E. minima* germinated.

Fruit set. The degree of fruit set resulting from intraspecific pollinations depended on the species and on the kind of pollination (Table 2). All artificial self-pollinations and nearly all artificial intraspecific cross-pollinations resulted in fruit set. The fruit set resulting from supported self-pollination compared with the fruit set resulting from artificial selfing and cross-pollination did not differ within the species *E. hirtella*, *E. minima* and *E. rostkoviana* but was significantly lower in *E. christii* ($p < 0.005$, Chi-square-test, Table 3, Fig. 4). Spontaneous selfing of ten *E. rostkoviana* plants and of eleven *E. christii* resulted in a clearly smaller fruit set (*E. rostkoviana*, 17 flowers, four fruit; *E. christii*, 16 flowers, one fruit). Fruit set following spontaneous selfing of *E. minima* and *E. hirtella* was 100%.

The fruit set resulting from the artificial interspecific crossing strongly depended on the species which were crossed and in all cases was significantly lower than the fruit set resulting from intraspecific crossing and selfing (*E. christii* x *E. hirtella* $p < 0.05$, *E. hirtella* × *E. christii* $p < 0.005$, Table 3, Fig. 4). No statis-

Table 3. Observed and expected fruit set following **a**) supported self-pollination and artificial selfing and crossing and **b**, **c**) following interspecific crossing and artificial selfing and crossing. Test values from the chi-square test

		Supported self-pollination		Artificial selfing and crossing	
		observed	Expected	observed	expected
a					
E. christii	No fruit-set	13	4.3	0	8.7
	Fruit-set	9	17.7	44	35.3
				Chi-square 29.3, df 1, $p < 0.001$	
E. minima	No fruit-set	1	0.4	0	0.6
	Fruit-set	22	22.6	33	32.4
				Chi-square 0.4, df 1, not sig	
E. rostkoviana	No fruit-set	2	1.1	1	1.9
	Fruit-set	17	17.9	30	29.1
				Chi-square 0.8, df 1, not sig	
b		*E. christii* × *E. hirtella*		Artificial selfing and crossing	
E. christii	No fruit-set	5	1.6	0	3.4
	Fruit-set	16	19.4	44	40.6
				Chi-square 9.7, df 1, $p < 0.01$	
c		*E. hirtella* × *E. christii*		Artificial selfing and crossing	
E. hirtella	No fruit-set	5	2	0	3
	Fruit-set	15	18	29	26
				Chi-square 7.05, df 1, $p < 0.001$	

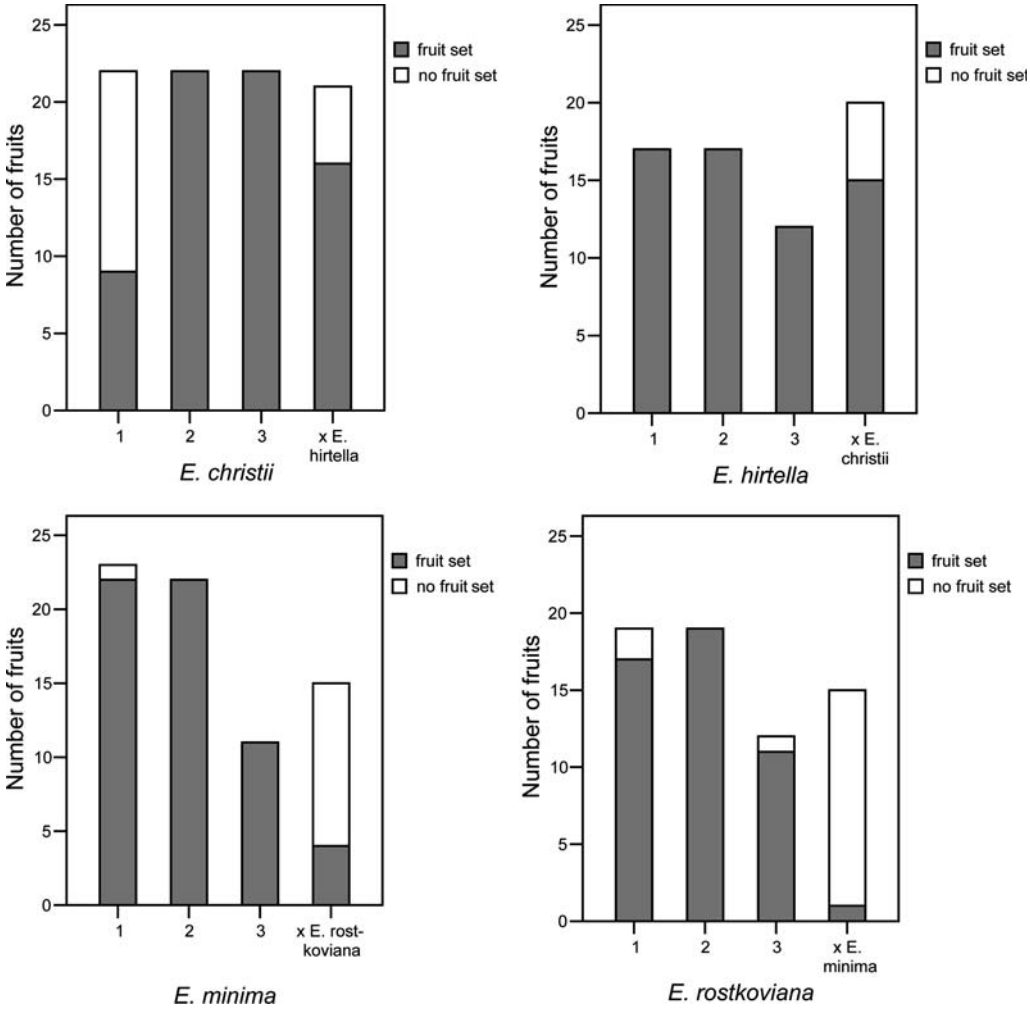

Fig. 4. Pollination effects on fruit set (P generation). (1) Supported self-pollination, (2) artificial self-pollination, (3) artificial cross-pollination. Grey fruit set, white no fruit set

tical test was applied to differences in fruit set in the diploid *E. rostkoviana* and the tetraploid *E. minima*, because 30 pollinations resulted in just five fruit (Fig. 4). Fruit set was much higher in the diploid species pair *E. christii* and *E. hirtella* (Fig. 4). The differences in the fruit set following reciprocal crosses (*E. hirtella* × *E. christii* 76.2%, *E. christii* x *E. hirtella* 80.0%) were not significant (Chi-square test, Table 4).

Seed set. Considerable seed set was observed in all fruit resulting from intraspecific pollinations (Fig. 5), except of fruit of *E. christii* and *E. rostkoviana*, resulting from spontaneous selfing.

The relative seed set (rss) after artificial selfing and crossing, respectively, was similar in all species. The same was true for the seed set after supported selfing and artificial selfing, except in the case of *E. christii*. In this species, the rss after supported self-pollination was significantly lower than the seed set after artificial selfing ($p < 0.05$, Wilcoxon signed-rank test, Table 5). In *E. christii* and *E. rostkoviana,* the rss after spontaneous selfing was distinctly smaller than the rss after supported self-pollination (*E. rostkoviana*, six seeds in three fruit; *E. christii*, one seed in one fruit). In both small-flowered species the seed

Table 4. Observed and expected fruit set resulting from reciprocal crosses

	E. christii × *E. hirtella*		*E. hirtella* × *E. christii*	
	observed	expected	observed	expected
No fruit set	5	5.12	5	4.88
Fruit set	16	15.88	15	15.12
			Chi-square 0.01, df 1, not sig.	

set was similar following spontaneous and supported selfing (spontaneous selfing *E. minima* 47.6%, E. hirtella 44.8%; supported selfing *E. minima* 47.0%, *E. hirtella* 56.0%).

Like the fruit set, the degree of the seed set in artificial interspecific crosses depended on the species which were crossed and was much higher in the diploid species pair *E. christii* and *E. hirtella* than in the species pair consisting of the diploid *E. rostkoviana* and the tetraploid *E. minima* (Fig. 5). Only one seed resulted from the pollinations of *E. rostkoviana* with *E. minima*. The contents of this seed was much smaller than its testa. One similar looking seed

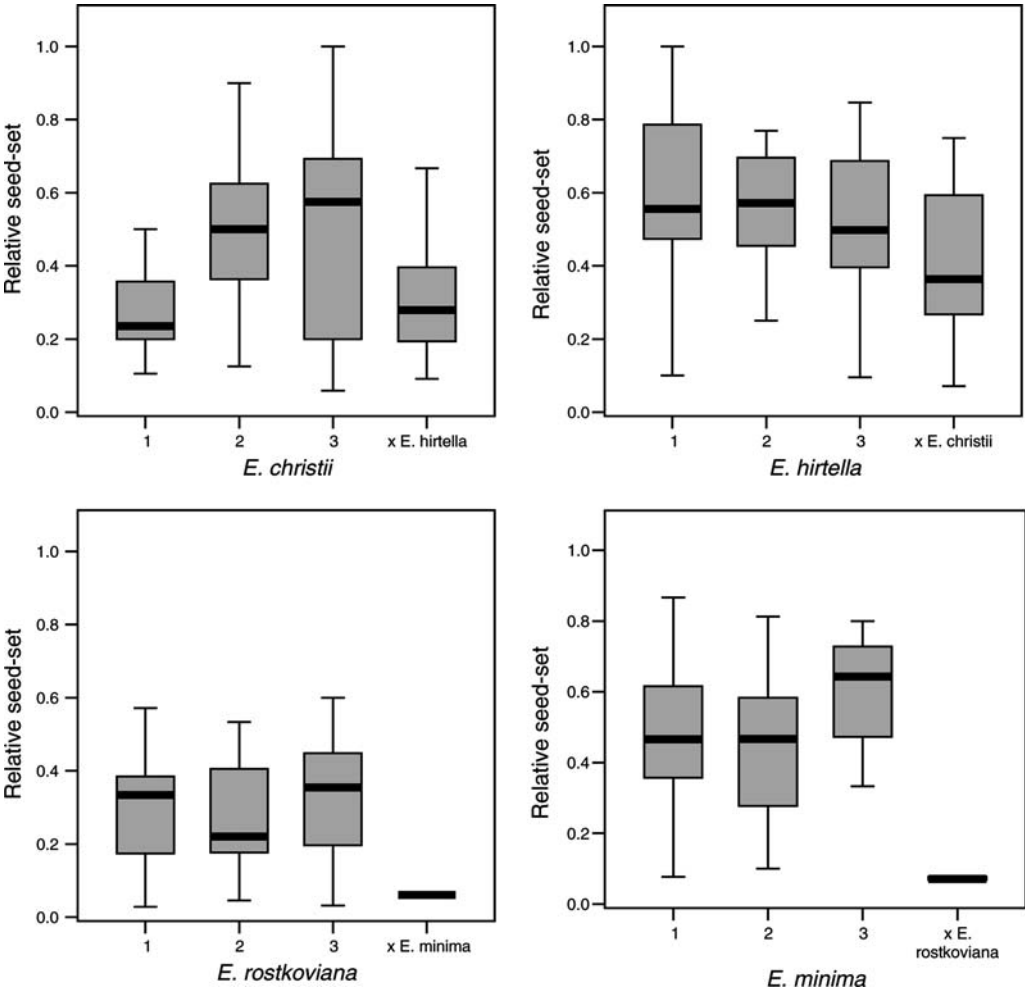

Fig. 5. Pollination effects on relative seed set (P generation). (1) Supported self-pollination, (2) artificial self-pollination, (3) artificial cross-pollination. The line inside the box marks the median, the lower and the upper hinge are the 25th and the 75th percentile, respectively. The whiskers include the range of the values

Table 5. Comparisons of the relative seed set resulting from supported and artificial self-pollination and from artificial self- and cross-pollination. Wilcoxon signed-ranks test, test statistic (asymptotic significance)

	E. christii	*E. hirtella*	*E. minima*	*E. rostkoviana*
Supported selfing/ artificial selfing	0.02	0.41	0.69	0.57
Artificial crossing/ artificial selfing	0.96	0.53	0.15	0.39

resulted from the reciprocal pollination, but the remaining four seeds resulting from this pollination did not differ from seeds resulting from intraspecific crossings.

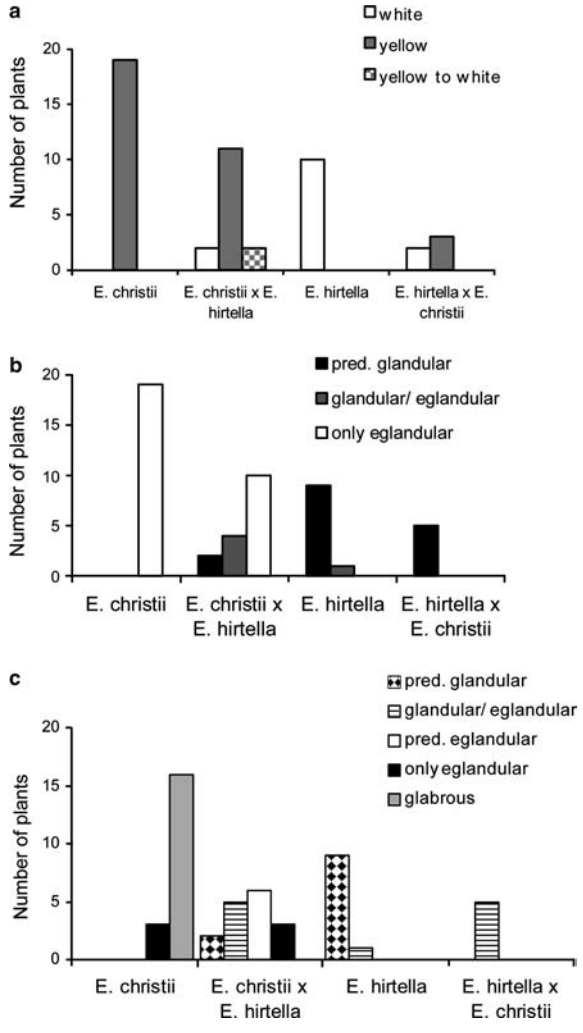

Fig. 6. Frequencies of qualitative morphological characters. (**a**) Colour of flowers, (**b**) indumentum of the stems, (**c**) indumentum of the leaves. Abbreviations according to Table 1

Morphological results. The characters that distinguish most clearly *E. christii* and *E. hirtella* are colour and size of the corollas, shape of the leaves and indumentum of stems and leaves. *Euphrasia christii* had large yellow flowers in contrast to the distinctly smaller, white flowers of *E. hirtella* (Fig. 6a, Table 6). The leaves of *E. christii* had cuneate leaf bases, were more than twice as long as they were wide and never bore glandular hairs. In contrast, the leaves of *E. hirtella* had cordate or rounded bases, were only 1.4 times as long as they were wide on average and, in addition to bearing a few eglandular hairs, were often densely covered with glandular hairs with multicellular stalks (Fig. 6c, Table 6). Referring to the indumentum, the stems behaved like the leaves (Fig. 6b, c). In both species, the coefficient of variation tended to be larger in leaf characters than in corolla characters (length of the upper lip, length of the tube, Table 6).

All three plants resulting from *E. christii* as maternal plant (F1) had yellow flowers, however, the flowers of one plant changed colour to white during the anthesis. Flowers of the F2 generation had yellow flowers much more frequently than white ones and flowers of a further plant changed colour to white. One hybrid resulting from *E. hirtella* as maternal plant (F1) had white and the hybrids of the F2 generation had either yellow or white flowers (Fig. 6a). The means of quantitative corolla characters were intermediate in both groups of hybrids (Table 6).

On the leaves of the hybrids, often short glandular hairs occurred together with short eglandular hairs, in contrast to the long-stalked glandular hairs occurring in *E. hirtella*.

Table 6. Means and coefficients of variation (CV) for quantitative morphological characters and indices. Abbreviations according to Table 1

Number of plants	*E. christii* $n = 19$		*E. christii* × *E. hirtella* $n = 16$		*E. hirtella* $n = 9$		*E. hirtella* × *E. christii* $n = 5$	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Flowers								
Upperlip	**8.1 mm**	8.8	**6.1 mm**	21.3	**4.7 mm**	10.5	**5.6 mm**	14.1
Tube	**5.7 mm**	11.8	**4.1 mm**	22.1	**3.3 mm**	13.4	**4.0 mm**	9.0
Calyx	**2.5 mm**	19.2	**2.4 mm**	16.0	**1.9 mm**	19.5	**1.7 mm**	22.4
In_calyx	**3.5**	20.5	**3.3**	26.3	**2.9**	29.6	**2.5**	10.2
Leaves								
In_leaf_c	**2.3**	18.6	**1.9**	18.8	**1.4**	13.9	**1.4**	15.5
Ang_l_c	**67.1**°	26.9	**114.8**°	29.0	**154.3**°	21.5	**172.4**°	26.0
Ang_t_c	**39.5**°	17.6	**53.7**°	29.5	**49.1**°	12.4	**63.4**	19.6
Teeth_c	**2.9**	22.7	**3.3**	28.6	**5.0**	17.3	**5.2**	25.1
In_leaf_fl	**2.5**	21.8	**1.7**	22.8	**1.4**	15.7	**1.3**	9.6
Ang_l_f	**66.9**°	28.9	**121.6**°	36.5	**165.7**°	24.3	**170.1**°	16.7
Ang_t_f	**40.2**°	23.0	**56.8**°	25.3	**52.0**°	21.7	**62.5**°	10.3
Teeth_f	**3.0**	27.2	**3.6**	24.4	**5.2**	18.6	**5.2**	21.1

The indumentum of the stems of the hybrids mostly resembled the indumentum of their maternal plants (Fig. 6b, c). The means of quantitative leaf characters were intermediate or were larger than the mean values of the parental plants (Table 6). Referring to quantitative characters, hybrids seem to be more similar to their maternal plant than to their paternal one (Fig. 7).

Discriminant analysis. Seventeen plants of *E. christii*, 14 plants of *E. christii* × *E. hirtella*, nine plants of *E. hirtella* and five plants of

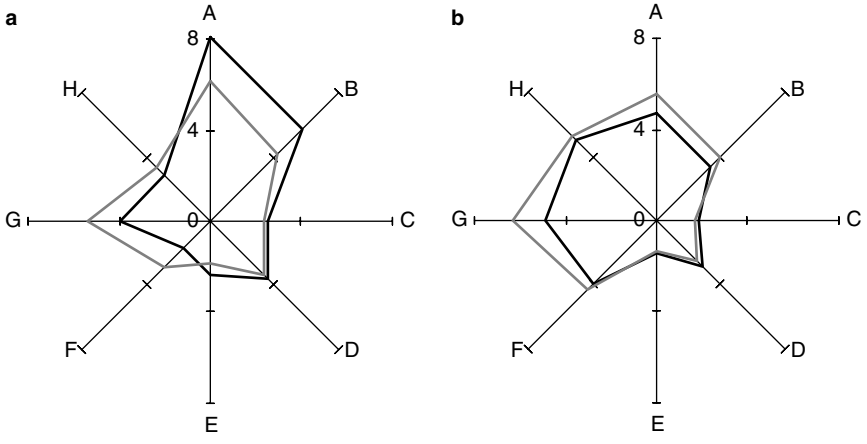

Fig. 7. Graphical representations of quantitative morphological characters and indices. (**a**) *E. christii* (black line) and *E. christii* × *E. hirtella* (grey line); (**b**) *E. hirtella* (black line) and *E. hirtella* × *E. christii* (grey line). (A) length upper lip, (B) length corolla tube, (C) length teeth calyx, (D) ratio of calyx length to calyx width, (E) ratio of leaf length to leaf width (cauline), (F) angle of leaf base (cauline, x 0.025), (G) angle of apex tooth (cauline, x 0.1), (H) number of leaf-tooth pairs (cauline)

Table 7. Standardised canonical discriminant function coefficients. The variables "number of teeth" and "index leaf length: leaf width" have been ln−transformed. Abbreviations according to Table 1

		Factor 1	Factor 2	Factor 3
Qualitative characters				
Corolla colour	white	0.34	−1.58	−0.64
	yellow	0.28	−0.28	−1.20
Stem hairs	pred glandular	0.66	−0.21	−1.21
	glandular/ eglandular	0.05	0.34	0.22
Leaf hairs	pred glandular	0.46	0.51	1.89
	glandular/ eglandular	1.76	1.45	0.63
	pred eglandular	1.62	1.08	0.89
	only eglandular	0.84	0.64	0.64
Quantitative characters				
Flowers	Upperlip	−1.09	−1.32	0.03
	Tube	−0.24	0.84	0.32
	Calyx	0.37	0.75	0.41
Leaves	Ang_l_c	0.81	−0.24	−0.41
	Ang_t_c	0.78	0.12	−0.01
	Teeth_c	−0.60	−0.85	0.14
	Ang_l_f	0.13	0.05	0.90
	Ang_t_f	−0.70	0.66	−0.16
	Teeth_f	−0.06	−0.13	−0.12
Indices				
Flowers	In_calyx	−0.85	−0.37	0.50
Leaves	In_leaf_c	−0.84	−0.79	−0.38
	In_leaf_fl	0.34	0.49	0.49
Canonical Correlation		0.99	0.95	0.91
Variation explained (%)		71.87	18.79	9.34

E. hirtella × *E. christii* were used in the canonical discriminant analysis. The assumptions "normality within groups" was tested using the Shapiro–Wilk's test and resulted in p values of > 0.05 (assumption of normal distribution accepted) except in two cases (number of teeth, index leaf length: leaf width). These two variables did not fit normal distribution, but ln-transformation minimised the deviations. Both variables were included in the analyses, being aware that the results of the subsequent test would have to be viewed with caution.

The discriminant analysis resulted in a highly significant separation of both the species and the hybrids. The first three canonical discriminant functions (CDF) contributed significantly to the separation of the four groups ($p < 0.000$) and were used in the analysis. The first two CDF's accounted for 90.7% of the variation. All individuals were classified correctly a posteriori in their respective groups.

In the first CDF, characters of the indumentum of the leaves and the size of the corolla were most powerful to separate the groups. In the second CDF the indumentum of the leaves, the colour of the flowers and the length of the corolla had the highest discriminatory power. Only 9.3% of the between-group variation was assigned to the third CDF; however, in this factor, the standardised canonical coefficient with the highest absolute value – a further qualitative leaf character – occurred (Table 7).

The scatter plot of the canonical discriminant analysis calculated from morphological traits is shown in Fig. 8.

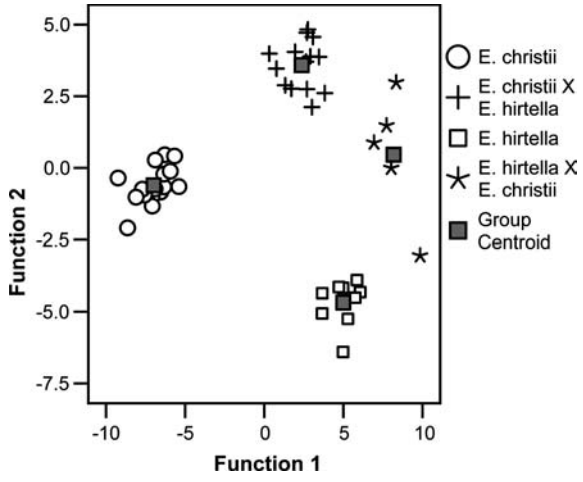

Fig. 8. Scatter plot of the canonical discriminant analysis for *E. christii*, *E. hirtella*, *E. christii* × *E. hirtella* and *E. hirtella* × *E. christii*

RAPD analyses. A total of 16 different RAPD fragments (ranging from 600–2200 base-pairs) which occurred either in *E. christii* or in *E. hirtella* were detected. The origin of the plants which were used to determine fragments typical for one of these species and the total sum of fragments is given in Table 8. While only three of seven fragments typical of *E. christii* occurred in all *E. christii* samples, seven of nine fragments typical of *E. hirtella* occurred in all *E. hirtella* samples. The frequencies of the fragments and the primers which were used in the analysis are given in Table 9.

In each of nine plants which resulted either from spontaneous selfing or from backcrossing of the F1 hybrids, between six and eleven RAPD fragments of both species occurred, proving the hybridogenous character of these plants. However, in one plant resulting from backcrossing (*E. hirtella* × (*E. hirtella* × *E. christii*)) none of the fragments typical of *E. christii* could be detected.

Ploidy analyses. The mean relative fluorescence in both *E. christii* (18 plants) and *E. hirtella* (6 plants) was 0.12 (± 0.02).

In the F1, flowcytometric analysis was done on seven plants of *E. christii*, four plants of *E. hirtella* and four hybrids (plants 8 and 9, *E. christii* × *E. hirtella*; plants 14 and 15, *E. hirtella* × *E. christii*). Both hybrids *E. hirtella* × *E. christii* (14 and 15) and one hybrid *E. christii* × *E. hirtella* (8) showed relative fluorescence similar to that of the parental species. In contrast, plant 9 showed a 1.8-fold relative fluorescence compared with the mean of the species (Fig. 9).

In the F2, flowcytometric analysis was done on eleven plants of *E. christii*, two plants of *E. hirtella* and 18 plants of hybridogenous origin. All 13 individuals resulting from the treatments 18 and 21 descended from plant 8 and 9 (F1). Except for one plant which resulted

Table 8. Source of the plants which were used for RAPD analyses and the number of RAPD fragments occurring in both species and in plants of hybridogenous origin. Treatments: 3 and 5 intraspecific crossing, 14 *E. christii* × (*E. christii* × *E. hirtella*), 25 *E. hirtella* × (*E. hirtella* × *E. christii*), 21 spontaneous selfing of *E. christii* × *E hirtella*

	Source of individual plants	n	Bands specific for	
			E. hirtella	*E. christii*
E. christii	Site; P generation; F1, resulting from treatment 3.	11	0	54
E. hirtella	Site; P generation; F1, resulting from treatment 5.	18	143	0
Plants of hybridogenous origin	F2, resulting from treatment 14, 21 and 25	10	58	35

Table 9. RAPD primers and frequencies of the fragments (%) in both species and in plants of hybridogenous origin

Primer	Fragment	*E. christii*	Frequency of fragments in %	
			E. hirtella	Plants of hybridogenous origin
B04	h1	0.0	100.0	80.0
B06	h3	0.0	100.0	77.8
B06	h4	0.0	100.0	77.8
B18	h1	0.0	100.0	88.9
C14	h1	0.0	100.0	60.0
C14	h2	0.0	100.0	70.0
D18	h1	0.0	44.4	50.0
D18	h2	0.0	100.0	90.0
D18	h3	0.0	55.6	30.0
B04	c1	100.0	0.0	50.0
B06	c2	66.7	0.0	22.2
B06	c3	66.7	0.0	60.0
B18	c2	100.0	0.0	22.2
B18	c3	100.0	0.0	88.9
C14	c1	45.5	0.0	60.0
D18	c1	80.0	0.0	50.0

from backcrossing of the hybrid (treatment code 18), they all resulted from spontaneous selfing of their maternal plants (code 21). The five remaining plants of hybridogenous origin either resulted from backcrossing of *E. hirtella* × *E. christii* or from backcrossing of *E. christii* × *E. hirtella* (treatment codes 14 and 25, Fig. 9).

At least one offspring of each of the plants 8 and 9 had a relative DNA content higher than that of its parents. The relative fluorescence of the progeny of plant 8 was similar to that of the maternal plant, except in the case of plant 14. The relative fluorescence of this individual was 1.5-fold the fluorescence of the maternal plant. The relative fluorescence of progeny of plant 9 (20, 24, 25) was 1.2, 1.1 and 1.5-fold the relative fluorescence of their maternal plant and twice to three times higher than the fluorescence of the species. The relative fluorescence of plants resulting from backcrossing (code 14, 18, 25) was within the range of the species (Fig. 9).

Traits of the putative tetraploid plants. The flowers of the polyploid plants tended to be smaller than those of the diploid hybrids. All flowers were initially yellow, but the flowers of two plants changed its colour to white during the anthesis. Except of the long leaf teeth, the polyploid hybrids were similar to *E. minima* in qualitative and quantitative characters (Table 10).

Discussion

Spontaneous and supported self-pollination. In the first part of this study, we used pollination experiments to test four *Euphrasia* species occurring in the Alps of Switzerland for self-compatibility and for their efficiency in self-pollination. The seed set following artificial selfing compared with the seed set following artificial cross-pollination was similar in each of the species, showing that all species were self-fertile. However, in the large-flowered species *E. rostkoviana* and *E. christii* sponta-

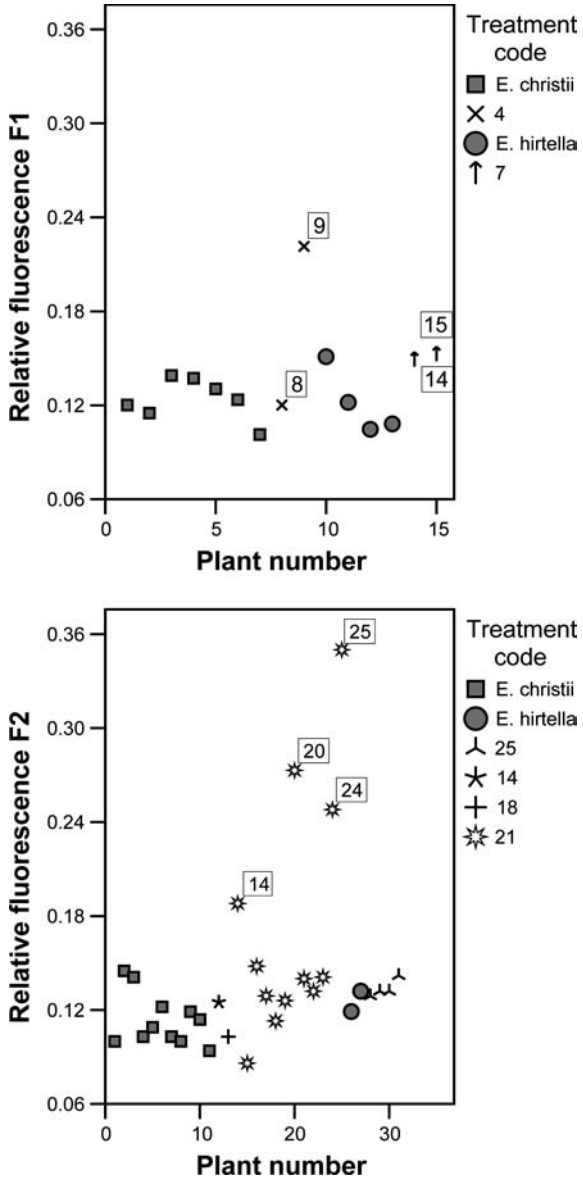

Fig. 9. Relative nuclear fluorescence of *E. christii* and *E. hirtella* and interspecific hybrids. Treatment codes refer to pollinations of the maternal plants. 4 *E. christii* × *E. hirtella*, 7 *E. hirtella* × *E. christii*, 25 *E. hirtella* × hybrid, 14 *E. christii* × hybrid, 18 hybrid × *E. christii*, 21 spontaneous selfing of hybrids (*E. christii* as maternal plant)

neous selfing is probably prevented by a combination of herkogamy and dichogamy. The success of spontaneous pollination in the small-flowered *E. minima* and *E. hirtella* strongly imply that in none of these species mechanisms occur which may prevent selfing.

Proterogyny and the distance between anthers and stigma led to assumptions that large-flowered *Euphrasia* species may not self or self only to a small degree (Müller 1881). However, based on flower observations alone, it is difficult to rate whether herkogamy, combined with dichogamy, may prevent selfing because by elongation of the corolla tube during anthesis, the positions of stigma and anthers alter and because dichogamy might be incomplete. Our pollination experiments allowed us to quantify the efficiency of self-pollination. Spontaneous selfing took place to a very small degree in *E. rostkoviana* and was prevented almost totally in *E. christii*. In contrast, flowers of plants which were moved during anthesis selfed successfully (supported selfing), and in *E. rostkoviana* supported selfing led to a seed set similar to that following artificial self-pollination. These results clearly indicate that herkogamy may prevent selfing in *E. rostkoviana* and *E. christii* almost totally. However, dichogamy must have been incomplete, because supported self-pollination resulted in seed set in both of the species. The differences between the two large-flowered species referring to the seed set following spontaneous selfing might be explained by a larger distance between anthers and stigma and/or by more complete dichogamy in *E. christii*. Assuming that changes of the flower positions, for example caused by wind, are common in natural habitats, it is to be expected that *E. rostkoviana* selfs to a high degree and *E. christii* only to a small degree, when no pollination by insects takes place.

Arctic and alpine annual species have been shown to have a high autodeposition efficiency (selfing in the absence of pollen vectors relative to that of open-pollinated control plants (Molau 1993)) and a high fruit set and seed:ovule ratio when pollinators are excluded (Gomez 2002). Selfing has been considered as reproductive assurance in habitats where conditions for outcrossing are unfavourable (Aarssen 2000). It is clear that annual species are more

Table 10. Characters of three polyploid hybrids. Values from *E. minima* (Hess et al. 1972, Yeo 1978) are added for comparison

Character	Hybrid 1 (F1)	Hybrid 2 (F2)	Hybrid 3 (F2)	*E. minima*
Colour of corolla	yellow	yellow	yellow/ white	yellow, white or lilac
Length corolla tube	3.4	3.0	3.7	2.0–4.0
Length upper lip	4.8	4.6	5.1	4.0–5.5(–6.0)
Branches	3	2	2	0–3(–5)
Stem hairs	eglandular	eglandular	eglandular	
Leaf hairs	short glandular	eglandular	short glandular	subglabrous to hairy, sometimes glandular
Leaf length caudal	0.83	0.85	0.89	
Leaf width caudal	0.36	0.47	0.41	
Number of leaf teeth caudal	4	5	3	1–4(–6)
Leaf length floral	0.56	0.57	0.67	0.3–1.2(–1.5)
Leaf width floral	0.38	0.44	0.42	
Number of leaf teeth floral	3	4	5	2–5(–6)

dependent on efficient pollination than perennial species. Field observations in arctic and alpine regions have shown that *E. minima* and other small-flowered *Euphrasia* species are rarely visited by insects (Müller 1881, Kerner von Marilaun 1888, Yeo 1966, Molau 1993, Kreisch 1996, Gomez 2002). Our pollination experiment clearly indicates that the small-flowered *E. minima* and *E. hirtella* are highly efficient selfers and are independent of pollinators. However, our results lead us to assume that the large-flowered *E. rostkoviana* – adapted to pollination by insects – has a selfing efficiency similar of that of *E. minima* and *E. hirtella*, but that selfing has to be supported by plant movements and probably occurs in the absence of pollinators. It is difficult to estimate the consequences of the low selfing efficiency of *E. christii*. Possibly, the large, bright yellow flowers may strongly attract insects, resulting in a high seed set when conditions are favourable and so compensating for a low seed set when insects are rare because of unfavourable meteorological conditions.

Artificial selfing and crossing The seed set following artificial selfing and crossing was similar not only in *E. minima*, *E. hirtella*, *E. christii* and *E. rostkoviana* but also in *E. willkommii*, a species probably related to *E. stricta* (Gomez 2002). However, the mean number of ovules per fruit which developed to seeds was higher when investigations were made in natural populations (*E. willkommii* 0.79, *E. frigida* 0.89 (Molau 1993, Gomez 2002)) and probably is higher in natural populations in the Alps (*E. minima* 0.78, own observation). A low seed-set may be caused by unsuitable pollination techniques and/or by environmental factors. Taking into consideration that the temperature in summer decreases by about 0.7 °C per 100 m altitude, the mean temperature in the botanical garden Zurich is about 10 °C higher than in the natural populations of the *Euphrasia* plants and was still higher in the glass houses. High temperatures together with caging after the pollinations might have influenced the surface and the receptivity of the stigmas or the germination of the pollen, leading to a lower seed set than has been observed in natural habitats of *Euphrasia*.

Interspecific crossing. As well as investigating the efficiency of spontaneous pollination, our study also focused on the seed set following interspecific crossing and on the fertility and morphological characteristics of the resulting offspring.

The large number of F2 plants resulting from selfing or back-crossing of *E. christii* × *E. hirtella* and *E. hirtella* × *E. christii* respectively and the large number of seeds obtained from selfing and back-crossing of the F2 plants demonstrated the fertility of the F1 hybrids and indicated the fertility of the F2 hybrids. The band patterns of the RAPD analysis proved without doubt the hybridogenous origin of nearly all the plants which we had investigated and provided evidence for the reliability of our pollination technique. In contrast to the successful interspecific crossing of *E. christii* and *E. hirtella*, we obtained only few normally developed seeds from the interspecific crossing of *E. minima* and *E. rostkoviana*. These results are in accordance with pollination experiments of species pairs alike and unlike in chromosome number done by Yeo (1966), except for the fact that we obtained some visually normal seeds from cross-pollination of the species pair unlike in chromosome number, possibly containing triploid embryos, but failing to germinate. Triploid hybrids probably resulting from interspecific crossing of *Euphrasia* species with different chromosome numbers have been found but seem to be rare in nature (Yeo 1956, Vitek 1986).

The relative nuclear fluorescence in flow cytometry of one of the F1 hybrids (*E. christii* × *E. hirtella*) led us to assume that this plant, or a part of it, was tetraploid. Our assumption was confirmed by the probably tetra- and pentaploid offspring of this plant, following spontaneous selfing. As far as we know, tetraploidy caused by interspecific crossing has not yet been found in studies investigating artificial interspecific crossings of *Euphrasia*. Polyploidization in the F1 generation following interspecific crosses may be caused by somatic doubling and has also been described as the union of unreduced gametes (Hahn et al. 1990, Ramsey and Schemske 1998). Although 2n gametes have been described in many plant taxa (Ramsey and Schemske 1998), we found no reference to 2n gametes, neither in *E. christii* nor in *E. hirtella*.

In contrast to Yeo (1956), who described a natural triploid *Euphrasia* hybrid, probably resulting from interspecific crossing of a diploid and a tetraploid *Euphrasia* species, we obtained a putative triploid plant from the spontaneous selfing of a diploid F1 hybrid. Diploid hybrids may produce triploid offspring by the union of an unreduced (2n) gamete with a reduced (1n) gamete. In contrast to the common claim that triploids are sterile, the results of several studies indicate that nonreduction and the production of 1n and 2n gametes enables allotriploids to produce tetraploids by selfing or back-crossing with a tetraploid species (Ramsey and Schemske 1998). In our study, spontaneous selfing of the triploid resulted in at least four probably normally developed seeds, indicating that the plant was not totally sterile. The tetraploid F1 and the triploid and tetraploid F2 plants indicate different pathways of polyploid formation following the interspecific crossing of *E. christii* and *E. hirtella*. Based on these results, it is reasonable to infer that polyploid formation in sympatric populations of *E. christii* and *E. hirtella* occurs if outcrossing takes place.

Polyploidization following interspecific crossing is assumed to be an important factor in the evolution of new species (Ramsey and Schemske 1998, 2002; Soltis et al. 2003) and might have played an important role in the evolution of tetraploid *Euphrasia* species. Based on ecological preferences and on morphological characters of *E. christii* and *E. hirtella*, Vitek (1986) discussed the descent of the tetraploid *E. minima* from preliminary forms of these two species. Morphological characters of three polyploid hybrids which we obtained from the hybridization of *E. christii* and *E. hirtella* (flower size and colour, indumentum of stem and leaves) were similar to those of *E. minima* and support the hypothesis of Vitek (1986).

The determination of the ploidy level by flow cytometry allowed us to screen large numbers of plants in a short time, but we did not succeed in optimising the analysis so that no peak shifting or the same degree of peak shifting occurred in all *Euphrasia* plants.

Therefore, we cannot exclude that some or all of the plants we referred to as tetraploids are aneuploid polyploids. Aneuploid polyploids as a rule survive and compete successfully with euploids. Furthermore, the fertility of early generation polyploids increases rapidly, owing largely to selection against meiotic configurations that generate unbalanced gametes (Ramsey and Schemske 2002). Therefore, we assume that for the establishment of a new tetraploid population from hybrids of *E. christii* and *E. hirtella*, it might be not relevant whether the parental hybrids were aneuploid or euploid polyploids.

To our knowledge, no evidence of interbreeding of *E. christii* and *E. hirtella* in natural populations has been found so far (possibly because the hybrids have been misidentified as *E. minima*, a highly variable species). The small-flowered *E. hirtella* is predominantly selfing and probably seldom visited by insects, but natural hybrids between small-flowered and between small- and large-flowered Euphrasia species, for example *E. salisburgensis* × *E. minima* and *E. hirtella* × *E. alpina*, indicate that a small flower size can not totally prevent interbreeding (Vitek 1985, Yeo 1978). Rare insect visits of *E. hirtella* might be the main factor for the lack of hybrids *E. christii* × *E. hirtella* in nature (if there is a lack), but other factors, for example a shortage of suitable ecological niches, may play an important role as well (Urbanska and Schütz 1986).

Morphology. Morphological characters clearly separate *E. hirtella* and *E. christii* and both hybrids. While the parental species are distinctly separated by flower colour and size or by the presence or absence of long glandular hairs respectively, a combination of predominantly qualitative characters improved the delimitation of the hybrids and their parents. Most useful for discrimination were the colour and size of the flowers and the indumentum of the leaves.

The canonical discriminant analysis resolved all four groups as distinct clusters. Along an axis connecting the centroids of *E. christii* and *E. hirtella*, both groups of hybridogenous origin were morphological intermediate between their parental species and were closer to their maternal progenitors than to the paternal species which points to maternal effects. However, both groups of hybrids were not clearly intermediate since they differed from both parental species along a direction perpendicular to the axis connecting the centroids of the species. Similar observations have been made by Emery and Chinnappa (1994) and Gugerli (1997). Emery and Chinnappa (1994) attributed these deviations to heterotic properties of the hybrids which are adaptively valuable for a diverse range of habitats.

The most striking characters which occurred in the plants of hybridogenous origin were short glandular hairs which did not occur in the parental species and flowers which changed their colour from yellow to white. Ontogenetic colour changes in fully turgid flowers are widespread throughout the angiosperms, but changing colour from yellow to white is a rare event. From about 400 colour-changing species enumerated by Weiss (1995), in only eleven species the whole flower or parts of it changed its colour from white to yellow. As far as we know, colour change has been described in only one species of *Euphrasia*: flowers of the Australian *E. glacialis* change their colour from yellow to orange. However, colour changing from yellow to white occurs in artificial hybrids of white-flowering *E. salisburgensis* and yellow-flowering *E. minima* and has also been detected in naturally mixed populations of these two species (own observations). In mixed populations of white- and yellow-flowering *E. minima*, we did not observe colour-changing plants so far.

Germination. The germination behaviour of seeds in a given alpine taxon may vary not only from year to year but also within a single individual, and the conditions under which seeds are produced sometimes influence not only the seed output but also germination (Urbanska and Schütz 1986). While the seeds of *E. christii*, *E. hirtella* and their hybrids

differed in their ability to germinate in the first spring after seed formation, none of the seeds germinated before March (about 80 days after sowing), showing innate seed dormancy. Seeds which failed to germinate in March or April did not germinate before having passed the whole growing season and the following winter. Similar behaviour has been observed not only in all the European *Euphrasia* species which have been investigated so far, but also in the alpine *Gypsophila repens* (Heinricher 1898, Yeo 1961, Urbanska and Schütz 1986). On the other hand, seeds of alpine *Euphrasia* species stored in a cold, dry place for about 15 months and sown in December, sometimes germinated within a week (own observation), survived the winter and flowered in the following summer. In natural populations of *Euphrasia*, we never observed seedlings in late autumn.

In natural habitats, the germination capacity of seeds is difficult to determine because predators might have eaten seeds or young seedlings or because no save site for successful germination might have been available. *E. hirtella* seeds sown in pots and stored in a cold frame in the Alpine Botanical Garden Schynige Platte (1960 m, Canton BE, Switzerland) germinated to 46 and 66% respectively (both n = 100). Seeds of the same sample, sown in a meadow at the same site, germinated to about 10% only (own observations). Seed germination in the second spring following seed formation was determined neither for the seeds collected in natural populations nor for the sowings at the Schynige Platte.

The germination of the seeds in the spring may be initiated by increasing day-length, increasing temperatures, a temperature sum or a combination of factors. However, seeds of *E. minima*, sown in December and stored in a refrigerator in the dark at constant temperatures of 5 °C, germinated at about the same time like seeds sown outside (own observation). To understand the germination behaviour of *Euphrasia*, further investigations are necessary.

References

Aarssen L. W. (2000) Why are most selfers annuals? A new hypothesis for the fitness benefit of selfing. Oikos 89: 606–612.

Bell K. L., Bliss L. C. (1980) Plant reproduction in a high arctic environment. Arctic Alpine Res. 12: 1–10.

Bingham R. A. (1998) Efficient pollination of alpine plants. Nature 391: 238–239.

Braun-Blanquet J., Rübel E. (1928) Flora von Graubünden 4.

Emery R. J. N., Chinnappa C. C. (1994) Morphological variation among members of the *Stellaria longipes* complex: *S. longipes*, *S. longifolia*, and *S. porsildii* (Caryophyllaceae). Pl. Syst. Evol. 190: 69–78.

Gomez J. M. (2002) Self-pollination in *Euphrasia willkommii* Freyn (Scrophulariaceae), an endemic species from the alpine of the Sierra Nevada (Spain). Pl. Syst. Evol. 232: 63–71.

Greilhuber J., Vitek E., Ehrendorfer F. (1984) Evolution alpiner Populationen von *Euphrasia* (Scrophulariaceae): Chromosomenzählungen an diploiden und polyploiden Sippen aus den Ostalpen. Pl. Syst. Evol. 144: 45–51.

Gugerli F. (1997) Hybridization of *Saxifraga oppositifolia* and *S. biflora* (Saxifragaceae) in a mixed alpine population. Pl. Syst. Evol. 207: 255–272.

Hahn S. K., Bai K. V., Asiedu R. (1990) Tetraploids, triploids, and 2n pollen from diploid interspecific crosses with cassava. Theor. Appl. Genet. 79: 433–439.

Hartl D. (1974) *Euphrasia*. In: Hegi G. (ed.) Illustrierte Flora von Mitteleuropa. 6. Carl Hanser, Berlin Hamburg, pp. 335–373.

Hegi G., Merxmüller H., Reisigl H. (1977) Alpenflora. Paul Parey, Berlin.

Heinricher E. (1898) Die grünen Halbschmarotzer, I. Jahrb. Wiss. Bot. 31: 77–124.

Hess H. E., Landolt E., Hirzel R. (1972) (eds.) Flora der Schweiz. 3. Birkhäuser, Basel, pp. 240–247.

Husband B. C., Schemske D. W. (1998) Cytotype distribution at a diploid-tetraploid contact zone in *Chamerion* (*Epilobium*) *angustifolium* (Onagraceae). Amer. J. Bot. 85: 1688–1694.

Käsermann C., Landergott U. (1999) Fundortaufnahmen von *Euphrasia christii*. SKEW.

Kearns C. A., Inouye D. W. (1993) Techniques for pollination biologists. University Press of Colorado, Niwot.

Kerner von Marilaun A. (1888) Ueber die Bestäubungseinrichtungen der Euphrasien. Verh. Zool.-Bot. Ges. Wien 38: 563–567.

Körner C. (1999) Alpine plant life. Functional plant ecology of high mountain ecosystems. Springer, Berlin Heidelberg New York.

Kreisch W. (1996) Vergleich der Polstervegetation in Hochgebirgen Mittel- und Nordeuropas aus blütenökologischer Sicht. 271. Cramer J., Berlin Stuttgart.

Lauber K., Wagner G. (1996) Flora Helvetica. Paul Haupt, Bern.

Molau U. (1993) Phenology and reproductive ecology in six subalpine species of Rhinanthoideae (Scrophulariaceae). Opera Bot. 121: 7–17.

Müller H. (1881) Alpenblumen, ihre Befruchtung durch Insekten und ihre Anpassungen an dieselben. Wilhelm Engelmann, Leipzig.

Ramsey J., Schemske D. W. (1998) Pathways, mechanism, and rates of polyploid formation in flowering plants. Annual Rev. Ecol. Syst. 29: 467–501.

Ramsey J., Schemske D. W. (2002) Neopolyploidy in flowering plants. Annual Rev. Ecol. Syst. 33: 589–639.

Reynolds D. N. (1984) Alpine annual plants: phenology, germination, photosynthesis, and growth of three Rocky Mountain species. Ecology 65: 759–766.

Richards J. A. (1996) Breeding systems in flowering plants and the control of variability. Folia Geobot. Phytotax. 31: 283–293.

Silverside A. J. (1991) The identity of *Euphrasia officinalis* L. and its nomenclatural implications. Watsonia 18: 343–350.

Soltis D. E., Soltis P. S., Tate J. A. (2003) Advances in the study of polyploidy since Plant speciation. New Phytol. 161: 173–191.

Urbanska K. M., Schütz M. (1986) Reproduction by seed in alpine plants and revegetation research above timberline. Bot. Helv. 96: 43–60.

Vitek E. (1985) Evolution alpiner Populationen von *Euphrasia* (Scrophulariaceae): *E. alpina* und *E. christii*. Pl. Syst. Evol. 149: 1–18.

Vitek E. (1986) Evolution alpiner Populationen von *Euphrasia* (Scrophulariaceae): Die tetraploide *E. minima*. Pl. Syst. Evol. 151: 241–269.

Vitek E., Kiehn M. (1990) Chromosome counts in *Euphrasia rostkoviana* (Scrophulariaceae) and related taxa. Flora 184: 31–41.

Von Wettstein R. (1896) Monographie der Gattung *Euphrasia*. Wilhelm Engelmann, Leipzig.

Weiss M. R. (1995) Floral color change: A widespread functional convergence. Amer. J. Bot. 82: 167–185.

Yeo P. F. (1956) Hybridisation between diploid and tetraploid species of *Euphrasia*. Watsonia 3: 253–269.

Yeo P. F. (1961) Germination, seedlings and the formation of haustoria in *Euphrasia*. Watsonia 5: 11–22.

Yeo P. F. (1966) The breeding relationship of some European Euphrasiae. Watsonia 6: 216–245.

Yeo P. F. (1968) The evolutionary significance of the speciation of *Euphrasia* in Europe. Evolution. 22: 736–747.

Yeo P. F. (1976) Artifical hybrids between some European diploid species of *Euphrasia*. Watsonia 11: 131–135.

Yeo P.F. (1978) A taxonomic revision of *Euphrasia* in Europe. Bot. J. Linn. Soc. 77: 223–334.

Addresses of the authors: Burgi Liebst (e-mail: liebst@systbot.unizh.ch), Jakob Schneller (e-mail: schnell@systbot.unizh.ch), Institut für Systematische Botanik, Universität Zürich, Zollikerstrasse. 107, 8008 Zürich, Switzerland.